Iraqi Journal of Cancer and Medical Genetics

Assessment of DNA damage in women using oral contraceptives by using comet assay

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Abstract:

This study includes the investigation of oral contraceptives pills effect (Yasmin) on women who used the contraceptive pills by measuring the percentage of DNA damage. Comparison process depends on evaluation of DNA damage percentage on blood lymphocytes in women who used the contraceptive pills for different periods by using comet assay. The presented results obtained from 25 women handling contraceptive pills and 15 women who had not used the pills.

The samples were divided into two groups according to the period of contraceptive pills treatment to (2-5) years and (6-8) years. Using image analysis software score comet, three parameters for measuring DNA damage were estimated to indicate it. DNA parameters were tail length, tail DNA % and tail moment. The results showed that the mean of tails lengths of comet in women who used birth control pills (2-5) years were (19.13%) while in women who used birth control pills (6-8) years were (25.08%). The observed values were significantly different ($P \leq 0.05$) from those in control, which were (0.8%). On the other hand, the percentage of DNA in the tail and tail moments of comet in women with contraceptive pills treatment (2-5) years were (20.12%) and (23.63%) respectively, while in women with contraceptive pills treatment (6-8) years were (28.63%) and (31.81%) respectively and the observed values were significantly different ($P \leq 0.05$) from those in control, which were (1.6%) of DNA in the tail and (0.01%) of tail moments. It is concluded from these results that the prolonged use of contraceptive drugs in our daily life will increase the percentage of DNA damage.

keyword: *DNA damage, Contracetives, Comet assay*

Introduction:

Contraception (birth control) is the process of prevents pregnancy by interfering with the normal process of ovulation, fertilization, and implantation. There are different kinds of birth control that act at different points in the process [1]. Birth control use in developing countries has decreased the number of maternal deaths by 40% and could prevent 70% if the full demand for birth control were met. Birth control also can improve adult women's delivery outcomes and the survival of their children by lengthening the time between pregnancies [2].

Oral contraceptives (OCs) are medicines taken by mouth to help prevent pregnancy. They are also known as the pills or birth control pills (BCPs) [3]. Most oral contraceptive pills contain a combination of estrogen (ethinylestradiol or mestranol) and progestin, but progestin-only preparations exist as well [4]. Combined oral contraceptive pills were developed to prevent ovulation by suppressing the release of gonadotropins. They inhibit follicular development and prevent ovulation as a primary mechanism of action [5].

Numerous herbs have been used historically to reduce fertility, and modern scientific research has confirmed antifertility effects in at least some of the herbs tested [6]. Women have used the seeds from Daucus carota for centuries as a contraceptive, the earliest written reference dates back to the late 5th or 4th century B.C. John Riddle writes in Eve's Herbs, that Queen Anne's lace seeds are one of the more potent antifertility agents available. The seeds harvested in the fall are a strong contraceptive if taken orally immediately after coitus [7]. Throughout history man has turned to nature for medicines. Microorganisms such as fungi offer a huge source of pharmaceutically useful molecules [8]. The ergot alkaloids (EA) are among the most important natural pharmaceuticals and toxins in human history that is derived from

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the fungus Claviceps purpurea [9].

Ergot extract has been used in pharmaceutical preparations, including ergot alkaloids (EA) in products such as Cafergot (containing caffeine and ergotamine [10]. EAs have been used for millennia to aid childbirth, birth control, treatment of migraines and, recently, treatment of Parkinsonism and other central nerve system disorders [11].

The comet assay (single cell gel electrophoresis) is used for the detection of primary DNA damage induced in isolated cells or nuclei from multiple tissues of animals usually rodents [12]. The main advantage of the comet assay is its simplicity. Minimal training is required for clinicians to conduct this assay and the equipment's for the assay are cheap and available [13].

The principle of the alkaline comet assay is based upon the ability of denatured, cleaved DNA fragments to migrate out of the nucleoid under the influence of an electric field, whereas undamaged DNA migrates slower and remains within the confines of the nucleoid when a current is applied. Evaluation of the DNA "comet" tail shape and migration pattern allows for assessment of DNA damage [14]. There are two conditions used in comet assay, alkaline and neutral conditions, neutral comet assay will detect mainly double-strand breaks and can be useful for assessing the DNA fragmentation associated with apoptosis [15].

Materials and Methods:

Alkaline comet assay (single cell gel electrophoresis) (SCGE)

Alkaline comet assay was used to investigate the possible DNA damage in peripheral blood lymphocytes from women who used contraceptive pills in comparison to healthy controls. The comet assay was performed under alkaline conditions [16].

This protocol describes the single cell gel electrophoresis assay (also known as the comet assay) which is a simple, rapid and sensitive technique for analysing and quantifying DNA damage in individual mammalian (and to some extent prokaryotic) cells. The more versatile alkaline method of the comet assay was developed by Singh and coworkers in 1988. This method was developed to measure low levels of strand breaks with high sensitivity.

The principle of the assay was based upon the cells which were embedded in a thin agarose gel on a microscope slide. The cells were lysed to remove all cellular proteins and the DNA was subsequently allowed to unwind under alkaline/ neutral conditions. Following unwinding, the DNA was electrophoresed and DNA was stained with a fluorescent dye. During electrophoresis, broken DNA fragments (damaged DNA) or relaxed chromatin migrates away from the nucleus. The extent of DNA liberated from the head of the comet is directly proportional to the DNA damage [17].

Preparation of reagents

Agarose preparation

Low and normal melting point agarose

Two water baths were equilibrated: one at 40ºC and other at ~100 ºC. by using 1% low melting point agarose was prepared by mixing powdered agarose (0.5g) with (50ml) distilled water in a glass beaker while 1.5% normal melting point agarose was prepared by mixing (0.75g) with (50ml) distilled water. Beakers were placed in the 100 ºC water bath (avoid vigorous boiling of the agarose and ensure that all agarose is dissolved). Then beakers were placed into a 40 ºC water bath.

Ethidium bromide Dye

Ethidium Bromide (EtBr; 10X Stock - 20 µg/ ml): 10 mg was added in 50 ml dH2O, and store at room temperature. For 1X stock -1 ml mixed with 9 ml dH2O.

Lysis buffer

Ingredients per 1000 ml: 2.5 M NaCl 146.1 g, 100 mM EDTA 37.2 g., and 10 mM Trise-base 1.2 g ingredients were added to about 700 ml distilled water and begin stirring the mixture. pH was adjust to 10.0 using 10 N NaOH and the volume was completed with distilled water to 890 ml , fresh 1% Triton X-100 and 10% Dimethylsulfoxide (DMSO) will increase the volume to the correct amount.

TBE electrophoresis Solution

Ingredients per 1000 ml: 10 mM Trise-base 10.8 g. and 100 mM EDTA 0.93g. The components were dissolved in 900 ml distilled water. The pH was adjusted to 13 (Alkaline pH) by using 10 N NaOH and the volume completed with distilled water to 1000 ml and kept at 4ºC until used.

Lysis of human peripheral blood RBCs:

The 10X RBC lysis buffer was diluted to 1X working concentration with distilled water. 1X solution was warmed to room temperature prior to use. 5 ml of 1X RBC lysis buffer was added to each tube containing whole blood. Gently vortex each tube immediately after adding the lysing solution. Tubes were incubated at room temperature and were protected from light for 10-15 minutes. Tubes were centrifuged at 1500 rpm for 2 min.

Preparation of samples and slides

1. Agarose slides were prepared by dipping the slides into normal molten 1.5 % (w/v) agarose.
2. Agarose was allowed to air dry to a thin film. Slides can be prepared ahead of a time and stored with a desiccant.
3. Slides were labeled on the end using a pencil, not a pen.
4. Cells suspension was centrifuged at 1500 rpm for 2 min. The supernatant was discarded and the pellet washed once with ice-cold PBS (without Mg2+ and Ca2+) and centrifuged at 1500 rpm for 2 min., then supernatant was discarded.
5. Cell sample was combined with low melting point agarose at 1:10 ratio (V/V) and the mixture (75µl/ well) immediately was added into slide comet by pipette.
6. The slides were hold horizontally then transferred to 4ºC in a dark container for 30 min.
7. The slide was transferred to a small basin containing pre chilled lysis buffer, the slide was immersed in the buffer overnight (18-20 h) at 4ºC in the dark.
8. After overnight, the slides were immersed with electrophoresis solution for 20min.

9. The slides were hold horizontally, and then transferred to a horizontal electrophoresis chamber filled with a cold TBE electrophoresis solution, 24volt (V) /cm and 300 (mA) was applied to the chamber for 18 min.
10. TBE electrophoresis solution was aspirated from chamber and replaced with Neutralisation Buffer, 0.4M of Tris-HCl solution (pH 7.5) for 5 min in order to neutralize the cells.
11. Diluted ethidium bromide dye 50 µl was added to each well of comet assay slide and incubated at room temperature for 15 min.
12. The slides were rinsed with distilled water to remove excess stain.
13. The slides were examined by fluorescence microscopy.

Results and Discussion:

The alkaline comet assay was used to evaluate the genotoxicity towards human peripheral blood lymphocytes. The DNA damage was quantified by measuring the displacement between the genetic material of the nucleus (comet head) and the resulting tail.

At least 50 randomly selected cells should be analyzed per sample. The quantification was done by using image analysis software comet score, the analysis software will calculate different parameters for each comet, and three parameters were estimated to indicate DNA migration, tail length (distance from the head center to the end of the tail), mean tail moment (appropriate index of induced DNA damage in considering both the migration of genetic material as well as the relative amount of DNA in the tail) and % DNA in tail [18].

The presented results of an alkaline comet assay on peripheral blood lymphocytes obtained from 25 women handling contraceptive pills and 15 controls. The samples were divided into two groups according to the period of contraceptive pills treatment to (2-5) years and (6-8) years, table (1).

Table (1): Results of alkaline comet assay on women peripheral blood lymphocytes.

Groups	Tail length	Tail moment	%DNA in tail
Negative control	**A** 0.8 ± 0.1	**A** 0.01 ± 0.003	**A** 1.6 ± 0.387
Contraceptive pills (2-5 years)	**B** 19.13± 0.01	**B** 23.63± 0.07	**B** 20.12± 0.03
Contraceptive pills (6-8 years)	**C** 25.08± 0.04	**C** 31.81± 0.18	**C** 28.63± 0.13

The mean of tails lengths of comet in women who used birth control pills (2-5) years were (19.13%) while in women who used birth control pills (6-8) years were (25.08%) and the observed values were significantly different (P≤0.05) from those in control subjects, which were (0.8%), table (1).
The percentage of DNA in the tail of comet in women who took contraceptive pills (2-5) years was (20.12%) while in women with contraceptive pills (6-8) years were (28.63%). The observed values were significantly different (P≤0.05) from those in control, which were (1.6%), table (1).

The tail moments of comet in women with contraceptive pills treatment (2-5) years were (23.63%) while in women with contraceptive pills treatment (6-8) years were (31.81%). The observed values were significantly different (P≤0.05) from those in control subjects, which were (0.01%), table (1), figure (1) and figure (2).
All the measures of DNA damage in peripheral lymphocytes taken from the women were significantly greater than in controls.

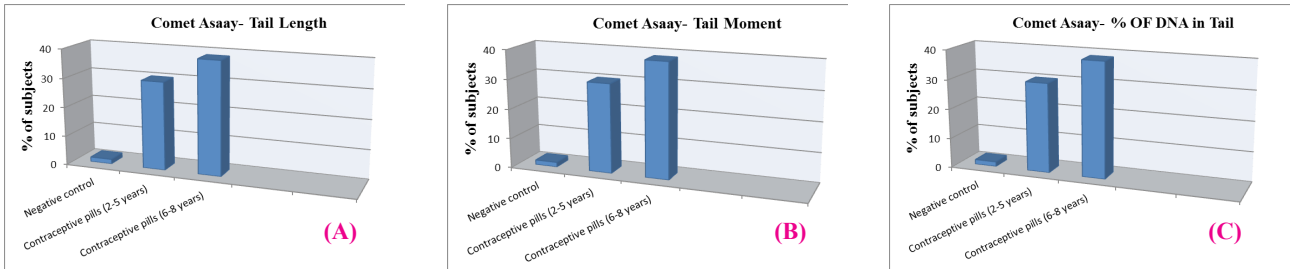

Figure (1): Histograms showing the distribution of the mean values for tail length (A), tail moment (B) and tail intensity (C) of comet in contraceptive pills treatment (2-5) years, contraceptive pills treatment (6-8) years and control.

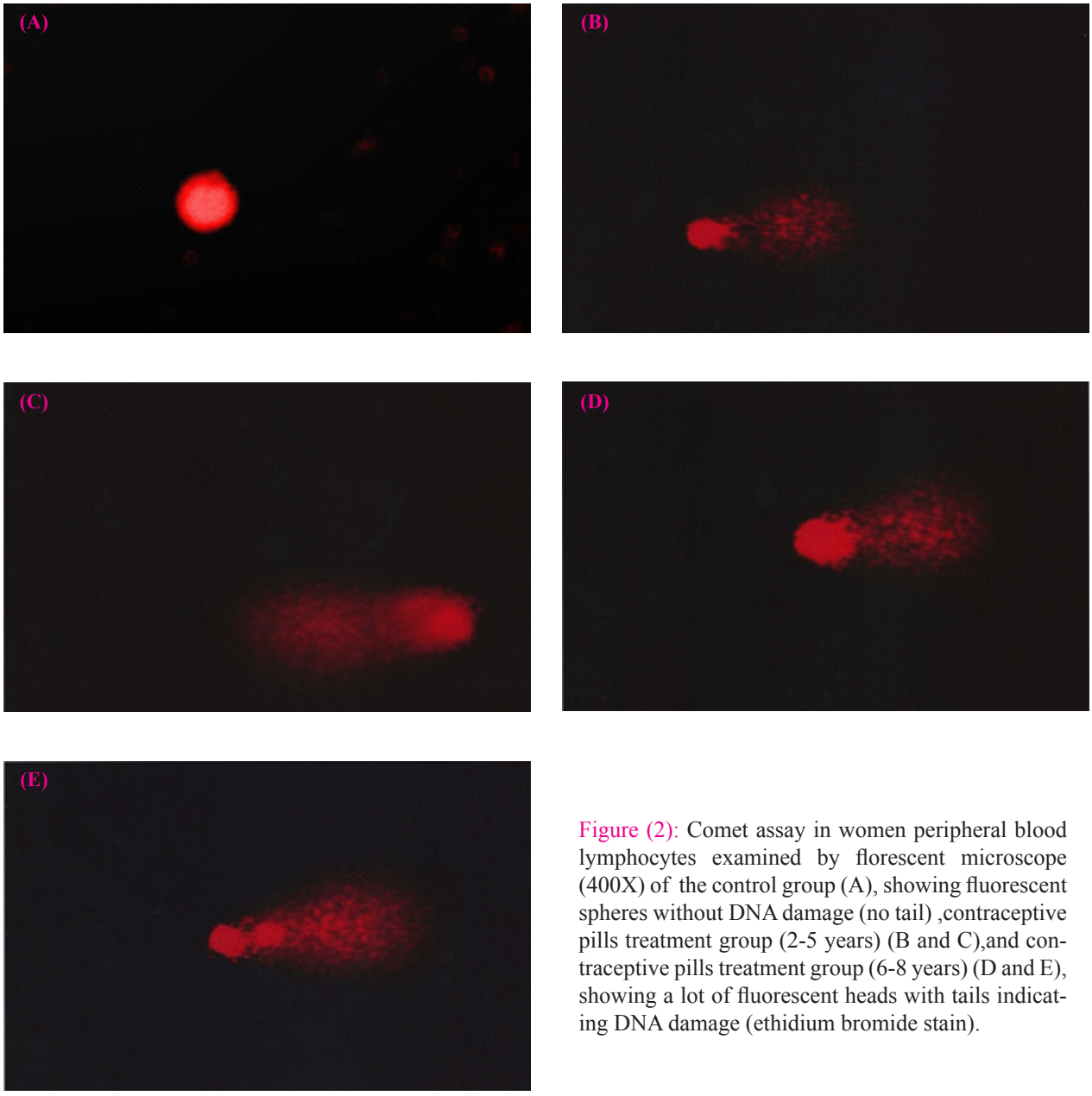

Figure (2): Comet assay in women peripheral blood lymphocytes examined by florescent microscope (400X) of the control group (A), showing fluorescent spheres without DNA damage (no tail) ,contraceptive pills treatment group (2-5 years) (B and C),and contraceptive pills treatment group (6-8 years) (D and E), showing a lot of fluorescent heads with tails indicating DNA damage (ethidium bromide stain).

The effect of the use of an oral contraceptive (OC) on the frequency of sister chromatid exchanges (SCEs) and on the response in the alkaline comet assay (single-cell gel electrophoresis) was investigated in 18 women taking contraceptive pills daily for 24 months [19]. A significant increase in the number of lymphocytes with DNA migration and an increased frequency of sister chromatid exchange per metaphase were observed in OC users as compared with their age-matched untreated controls. As higher incidences of spontaneous SCEs in peripheral blood lymphocytes have been reported to occur in females during pregnancy due to profound changes in the levels of certain sex hormones such as progesterone and estrogen, particularly during the last trimester, 17 pregnant women served as positive controls in this study in order to test the rate of genetic damage due to those changes. Higher frequencies of SCEs and comet responses were observed in pregnant women than in their matched controls. However, no statistically significant difference in DNA damage was observed between OC users and pregnant women.

The fact that prolonged and extensive use of these drugs in our daily life may be hazardous and also, that OC users should be aware of multifactorial risk factors (environmental, genetic and life style patterns) that may be responsible for additional DNA damage.

References:

1. San Diego, C. A. (2003). Birth control pills: A medical dictionary, bibliography and annotated research guide to internet references icon health publications.
2. Ahmed, S.; Li, Q.; Liu, L. and Tsui, A. O. (2012). Maternal deaths averted by contraceptive use: An analysis of 172 countries. Lancet, 380 (9837):111–125.
3. Siberry, G. K.; and Robert, I. (2000). The Harriet Lane Handbook , 15th ed. Philadelphia, Mosby.
4. Wilson, N. M.; Laursen, M. and Lidegaard, O. (2012). Oral contraception in Denmark 1998-2010. Acta Obstet. Gynecol. Scand, 91(7):810-815.
5. Nelson, A. L.; Cwiak, C. (2011). Combined Oral Contraceptives (COCs). In Hatcher, R. A.; Trussell, J.; Nelson, A. L.; Cates, W. Jr.; Kowal, D. and Policar, M. S. (eds.). Contraceptive Technology (20th ed.). New York, Ardent Media. pp. 249–341.
6. Umadevi, M.; Sampath Kumar, P. K.; Debjit, B. and Duraivel, S. (2013). Medicinal plants with potential antifertility activity. J. of Med. Plants Studies. 1(1): 26-33.
7. Riddle, J. M. (1997). Eve's Herbs - A History of Contraception and Abortion in the West. Cambridge, Ma. Harvard University Press
8. Langley, D. (1998).Exploiting the fungi: novel leads to new medicines. Mycol., 11: 165-166.
9. Matossian ,M. K. (1989). Poisons of the Past: Molds, Epidemics, and History, Yale University Press, New Haven. CT.
10. Black, M. H. and Halmer, P. (2006). The Encyclopedia of Seeds: Science, Technology and Uses. CABI, Wallingford, UK. P. 226.
11. Di Menna, M. E.; Finch, S. C.; Popay, A. J. and Smith, B.L. (2012). A review of the Neotyphodium lolii/Lolium perenne symbiosis and its associated effects on animal and plant health, with particular emphasis on ryegrassstaggers. Vet. J., 60:315–328.
12. Rothfuss, A.; O'Donovan, M.; De Boeck, M.; Brault, D.; Czich, A.; Custer, L.; Hamada, S.; Plappert-Helbig, U.; Hayashi, M.; Howe, J.; Kraynak, A.R.; van der, L. B-J.; Nakajima, M.; Priestley, C.; Thybaud, V.; Saigo, K.; Sawant, S.; Shi, J.; Storer, R.; Struwe, M.; Vock, E. and Galloway, S. (2010). Collaborative study on fifteen compounds in the rat-liver comet assay integrated into 2- and 4-week repeat-dose studies. Mutat. Res., 702:40-69.
13. Collins, A. R.; Oscoz, A. A.; Brunborg, G.; Gaivão, I.; Giovannelli, L.; Kruszewski, M.; Smith, C. C. and ttina, R. (2008). The comet assay: topical issues. Mutagenesis. 23:143-151.
14. Lemay, M. and Wood, K.A. (1999). Detection of DNA damage and identification of UV induced photoproducts using the CometAssay® kit. Biotechniques, 27(4):846-851.
15. Zainol, M.; Stoute, J.; Almeida, G. M.; Rapp, A.; Bowman, K. J. and Jones, G. D. (2009). Introducing a true internal standard for the comet assay to minimize intra- and inter-experiment variability in measures of DNA damage and repair. Nucleic Acids Res., 37(22): 150.
16. Peggy, L. and Judit, P. B. (2006).The comet assay: a method to measure DNA damage in individual cells. Brit. Columbia Cancer Res.h Center, 1(1)23-29.
17. Lovell and Omori. Statistical issues in the use of the comet assay (2008). Mutagenesis, 2 (3):171-182.
18. Azqueta, A.; Shaposhnikov, A. and Collins, A. (2009). Mutation research genetic toxicology and environmental mutagenesis. Mutat. Res., 674:101-108.
19. Biri, A.; Civelek, E.; Karahalil, B. and Sardas, S. (2002). Assessment of DNA damage in women using oral contraceptives. Mutat. Res., 521:113–119.

تقيم الضرر الحاصل في الدِنا للنساء المستخدمات لحبوب منع الحمل بواسطة اختبار المذنب

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الخلاصة:

هدفت الدراسة الحالية الى معرفة تأثير حبوب منع الحمل (ياسمين) على النساء اللواتي يستخدمن الحبوب من خلال قياس نسبة الضرر الحاصل في الـ DNA. وقد استندت عملية المقارنة على حساب نسبة الضرر الحاصل في الـ DNA على خلايا الدم اللمفاوية عند النساء اللواتي يستخدمن الحبوب ولفترات مختلفة باستخدام فحص اختبار المذنب. حيث تم الحصول على النتائج المقدمة من 25 عينة دم من النساء اللاتي يستخدمن الحبوب و 15 عينة اخرى كمعاملة سالبة. و قد تم تقسيم عينات الدم اعتمادا على مدة الاستعمال لـ (2-5) سنوات و (6-8) سنوات. تم تقيم الضرر الحاصل في الـ DNA باستخدام image analysis software score comet. حيث كانت معايير الـ DNA المعتمدة لمعرفة نسبة الضرر هي tail length, tail DNA % و tail moment وقد بينت النتائج ان نسبة tail length للنساء اللواتي يستخدمن الحبوب للفترة من (2-5) سنوات كانت (19.3%) بينما عند النساء اللواتي يستخدمن الحبوب للفترة من (6-8) سنوات كانت (25.08%) والنتائج الملحوظة ادت الى زيادة معنوية ($P\leq0.05$) بالمقارنة مع المعاملة السالبة والتي كانت (0.8%). من جانب اخر كانت tail DNA % وtail moment لفترة الاستخدام من (2-5) سنوات (20.12%) و 23.63%)) على التعاقب, ولفترة الاستخدام من (6-8) سنوات كانت ((28.63% و (31.81%) على التعاقب والقيم الملحوظة تمثل زيادة معنوية ($P\leq0.05$) اذا ما قورنت مع المعاملة السالبة والتي كانت (1.6%) بالنسبة لـ tail DNA % و (0.01%) بالنسبة لـ tail moment. من هذه النتائج نستدل على ان طول فترة الاستخدام لعقارات منع الحمل في حياتنا اليومية سوف تزيد من نسبة حصول الضرر في الـ DNA.

البحث مستل من اطروحة دكتوراه للباحث الاول